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Effectiveness of Carvacrol Derived from *Thujopsis dolabrata* var. *hondai* Sawdust against *Thecodiplosis japonensis* (Diptera: Cecidomyiidae)

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Abstract: The larvicidal component from sawdust of *Thujopsis dolabrata* var. *hondai* (Family Cupressaceae) against the pine needle gall midge (*Thecodiplosis japonensis*) was isolated by chromatographic techniques and characterized by spectral analysis as carvacrol. In a laboratory study using the impregnated filter paper method, carvacrol was more toxic to *T. japonensis* larvae than β-thujaplicine, cedrol, α-terpinol, thujone or thymol. In field studies with soil injections of carvacrol, this compound exhibited potent larvicidal activity, suggesting that this activity might be attributable to fumigant action. In a test with trunk implantation, a mixture of carvacrol and phosphamidon ($0{\cdot}15 + 0{\cdot}15$ ml cm^{-1} diameter at breast height) revealed much more potent larvicidal activity than phosphamidon alone ($0{\cdot}3$ ml cm^{-1} DBH) in spite of little or no larvicidal activity of carvacrol alone ($0{\cdot}3$ ml cm^{-1} DBH), indicating a possible synergistic effect. As a naturally occurring insecticide, carvacrol could be useful as a new preventive agent against damage caused by *T. japonensis*.

Key words: *Thecodiplosis japonensis*, *Thujopsis dolabrata* var. *hondai*, carvacrol, larvicidal activity, trunk implantation

1 INTRODUCTION

The pine gall midge (*Thecodiplosis japonensis* Uchida & Inouye) is one of the most serious insect pests of pines in Korea.[1] In the spring (May and June), just as the leaf buds swell, emerging adults deposit eggs on developing needles. After hatching, young larvae crawl down to the leaf sheath and feed by sucking sap, resulting in the formation of galls. Large numbers of galls on pine trees cause premature defoliation which results in simultaneous retardation in both terminal and cambial growth of the tree.[2–4] In 1995, approximately 212 000 ha of red pines (*Pinus densiflora* Sieb. et Zucc.) and black pines (*P. thunbergii* Parl.) were infested by this species.[5]

Control is primarily dependent upon repeated applications of soil insecticides against *T. japonensis* larvae in March to April and/or October to November and trunk implantation of systemic insecticides such as phosphamidon in mid-June.[5] Although they have effectively controlled this gall midge species, their extensive use for the past decades has led to the development of resistance to insecticides, adverse effects on non-target organisms, environmental problems and human health hazards. Besides these problems, factors such as labour and insecticide costs should be considered. This economic consideration and increasing concern over possible adverse effects of the earlier types of insecticide have brought about the need for the development of new types of selective alternatives or biorational management methods without, or with reduced, use of conventional insecticides.

Plants may provide alternatives to currently used insect control agents, because these are often active against a limited number of species, including specific target insects, are often biodegradable to non-toxic products, and potentially suitable for use in IPM. Therefore, much effort has been focused on plant-derived

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materials for potentially useful products as commercial insecticides or as lead compounds.[6–8] We reported in a previous paper[9] that, among 190 plant species, crude oil of *Thujopsis dolabrata* (L.) Sieb. & Zucc. var. *hondai* sawdust had potent larvicidal activity against *T. japonensis*.

In the laboratory and field studies described herein, we assessed the effectiveness of the *Thujopsis* sawdust-derived materials against *T. japonensis*.

2 MATERIALS AND METHODS

2.1 Laboratory studies

2.1.1 Insects

Twigs of red pine trees severely infested with *T. japonensis* at Mikum-Si (Kyungi Province) were collected on 13 September 1994, and brought to the laboratory. The gall-formed needles only were selected and cut by a razor, and then larvae in the galls were carefully collected with a camelhair brush in Petri dishes (11 cm dia.) containing filter paper (Toyo No. 2; 6 cm dia.) moistened with 3 ml distilled water.

2.1.2 Chemicals

Chemicals used in this study were as follows: β-Thujaplicine and α-terpineol (ExtraSynthese, France), cedrol (Karlsruhe Roth, Germany), thujone and carvacrol (Tokyo Kasei, Japan) and thymol (Wako, Japan). Phosphamidon 500 g litre^{-1} SL was supplied by Han Kook Sam Gong (Seoul, Korea). All other chemicals were of reagent grade.

2.1.3 Isolation and identification

The sawdust of *T. dolabrata* var. *hondai* was obtained from Taiyo Kagaku Central Laboratories, Yokkaich, Mie Prefecture, Japan. Purification and isolation of insecticidal component(s) from the steam distillate of *Thujopsis* sawdust active against *T. japonensis* larvae was performed as previously described.[10] The fractions and isolates dissolved in methanol were bioassayed at a rate of 10 mg per paper by the impregnated filter paper method (Section 2.1.4).

Structural determination of the active isolate was made by spectroscopic analysis. [^{1}H] and [^{13}C]NMR spectra were recorded in deuterochloroform with a JEOL GSX-400 FT-NMR (400 MHz) spectrometer. UV spectra were obtained with a Unikon 942 spectrophotometer, IR spectra on a Biorad FT-80 spectrometer and EI-MS spectra on a JEOL JMS-AX 505 spectrometer.

2.1.4 Bioassay

Larvicidal activities of the *Thujopsis* sawdust-derived materials against *T. japonensis* were tested by the impregnated filter paper method. Appropriate doses of the materials dissolved in methanol were applied to filter papers (Toyo No. 2; 6 cm dia.) by syringe. After evaporation, larvae were placed on to the papers in individual Petri dishes (6 cm dia.). Controls received methanol. All treatments were conducted in triplicate, and 20 larvae were used in each assay. Treated larvae were held in a room at 25($\pm$1)°C, 50–60% RH under a 16 : 8 h light : dark cycle. Mortalities were determined 48 h after treatment. Data from all bioassays were corrected for control mortality using Abbott's formula.[11]

The larvicidal activity of the most active isolate was examined and compared with that of the *Thujopsis*-derived terpenoids (β-thujaplicine, cedrol and α-terpineol) and two commercial terpenoids (thujone and thymol) as previously described.[10]

2.2 Field studies

2.2.1 Soil application

Carvacrol efficacy was evaluated by soil injection at damaged sites of Mikum-Si (Kyunggi Province) and Kangnung-Si (Kwangwon Province) on 20 May and 25 November 1994, respectively. Three replicate plots of each of the treatments plus three checks, each 5 × 5 m, were laid out in a completely randomized block. The number of larvae per plot in the soil was determined before treatment. Injections of carvacrol were made 10 cm deep with an injector at 30-cm intervals in rows 30 cm apart at rates of 1, 2·5 and 5 ml. After injection, the soil was tamped by stamping. Polyethylene traps (20 cm dia.) for emerging midge adults were placed on the treated and untreated soils (Fig. 1). These traps were placed 1·5 m apart in the fields at Mikum-Si and Kangnung-Si on 31 May 1994 and on 1 June 1995, respectively. Traps were checked twice weekly before capture of the first midge adults and every day thereafter. Midge adults were removed from the traps and counted.

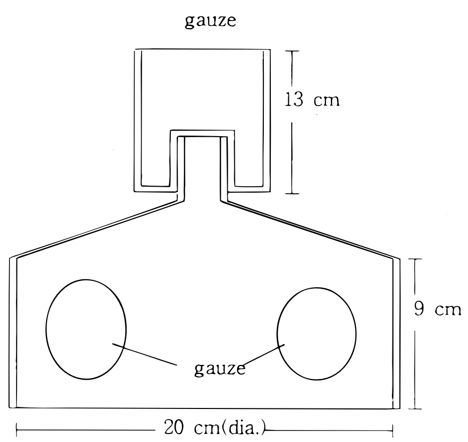

Fig. 1. Polyethylene trap for emerging *Thecodiplosis japonensis* adults.

Emergence rate was calculated as (total number of midge adults captured at each trap for 30 days after treatment/number of gall midge larvae in soil before treatment) × 100. However, efficacy was expressed as protective value (PV) using the formula

$$PV = [1 - (U_n - U_e/U_n)/1 - (T_n - T_e/T_n)] \times 100,$$

in which U_n = number of gall midge larvae in untreated plot before treatment; U_e = total number of midge adults captured at each trap in untreated plot after treatment; T_n = number of gall midge larvae in treated plot before treatment and T_e = total number of midge adults captured at each trap in treated plot after treatment.

2.2.2 *Trunk implantation*

All treatments were conducted on uniform 20- to 25-year-old red pine stands (*c*.12–15 cm dia. and 5–6 m high) at Pyungchang-Gun and Kangnung-Si (Kangwon Province) on 15 June 1994 and on 20 June 1995, respectively. Phosphamidon alone and in mixture with carvacrol was applied as trunk implantations to mature five-tree plots in a completely randomized block with three replications. Carvacrol was introduced directly into the trees, while phosphamidon was introduced as a commercial 500 g litre SL. Implantations were made with a motor-driven injector (Kawasaki TD33D, Japan) after drilling holes (1 cm dia.) in the trunk at 60 cm above soil level. The holes were drilled 8 cm deep and at 45° to the main axis of the tree. Control trees were drilled without the introduction of chemical. Carvacrol, phosphamidon and carvacrol + phosphamidon (1 + 1 by volume) were applied at 0·3 ml per cm of trunk diameter at breast height (DBH). In a preliminary test, little or no larval mortality occurred in pine trees treated with 0·15 ml cm^{-1} DBH.

The treatments at Pyungchang-Gun and Kangnung-Si were sampled at random from each treatment on 20 September 1994 and 22 September 1995, respectively. The gall-formed needles from twigs of the red pine trees were cut with a razor, and larvae in the galls which failed to move when prodded with a camelhair brush were considered dead.

To determine phytotoxic effect, the presence or absence of leaf burn and damage in treated trees was assessed after each chemical application and compared with that of the untreated trees.

2.3 Statistical analysis

The percentage mortality and emergence rate were determined and transformed to arcsine values for analysis of variance. Means(±SEM) of untransformed data were reported. Treatment means were compared and separated by Tukey's studentized range test at $P = 0{\cdot}05$.[12]

3 RESULTS

3.1 Laboratory studies

The crude oil of the *Thujopsis* sawdust revealed potent larvicidal activity against *T. japonensis* and was separated into four fractions, using a centrifugal thin-film evaporator. The fractions and isolates dissolved in methanol were bioassayed by the impregnated filter paper method (Table 1). At 10 mg per paper, fractions **I** and **II** caused 100 and 58% mortalities against these larvae, respectively. Purification of the biologically active component(s) from the most potent fraction **I** was done by silica gel column chromatography and HPLC.

Bioassay-guided fractionation and HPLC of the *Thujopsis* crude oil afforded an active compound which was characterized as the terpenoid carvacrol by spectroscopic methods including MS and NMR, and comparison of the spectral data with literature.[10] The larvicidal activity of carvacrol was compared with that of other *Thujopsis*-derived and commercial terpenoids (Fig. 2) as previously described[10] and the data are presented in

TABLE 1
Larvicidal Activity of the Crude Fractions from *Thujopsis dolabrata* var. *hondai* Sawdust against *Thecodiplosis japonensis* Larvae by Impregnated Filter Paper Bioassay

Fraction[a]	*Mortality* (%) (±*SEM*)[b]
I	100a
II	58·3 (±4·4)b
III	26·7 (±3·3)c
IV	10·0 (±0·0)d

[a] Rate = 10 mg per paper (6 cm dia.).
[b] Means followed by the same letter in column are not significantly different ($P = 0{\cdot}05$; Tukey's studentized range test) (20 larvae per replicate; three replicates per treatment: $n = 60$).

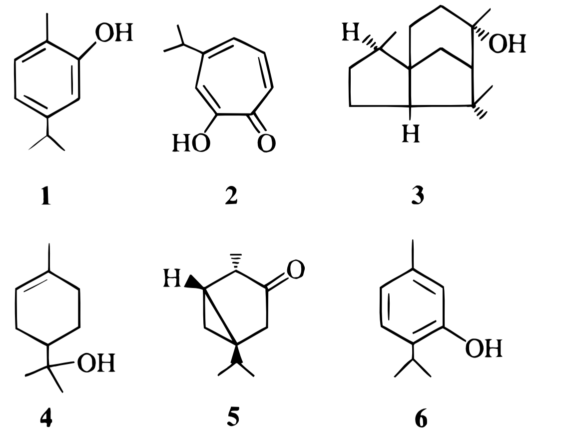

Fig. 2. Structures of compounds tested. **1** Carvacrol; **2** *β*-Thujaplicine; **3** Cedrol; **4** α-Terpineol; **5** Thujone; **6** Thymol.

TABLE 2
Larvicidal Activity of *Thujopsis dolabrata* var. *hondai*-derived Materials and Commercial Terpenoids against *Thecodiplosis japonensis* Larvae by Impregnated Filter Paper Bioassay

Compound	*Mortality* (%) (±*SEM*)[a]		
	1[b]	*5*[b]	*10*[b]
Carvacrol	75·5 (±2·3)a	79·0 (±3·8)a	93·3 (±1·7)a
β-Thujaplicine	67·3 (±3·7)ab	70·0 (±5·8)a	73·3 (±4·4)a
Cedrol	59·3 (±5·8)abc	70·0 (±5·8)a	70·0 (±7·6)a
α-Terpineol	52·7 (±6·4)abc	55·0 (±5·0)a	70·0 (±7·6)a
Thujone	47·7 (±3·9)bc	65·0 (±5·0)a	67·7 (±4·3)a
Thymol	42·3 (±6·2)c	60·0 (±5·8)a	76·3 (±4·5)a

[a] Means followed by the same letter in column are not significantly different ($P = 0{\cdot}05$; Tukey's studentized range test) (20 larvae per replicate; three replicates per treatment: $n = 60$).
[b] Rate = mg per paper (6 cm dia.).

TABLE 3
Effect of Carvacrol on Emergence Rate of *Thecodiplosis japonensis* Larvae by Soil Injection

Rate[a] (*ml*)	*Experiment I*[b]		*Experiment II*[c]	
	ER[d] (%) (±*SEM*)[e]	*PV*[f] (%)	*ER* (%) (±*SEM*)	*PV* (%)
0	84·8 (±4·1)a		84·0 (±3·2)a	
1	—[g]		33·7 (±3·6)b	59·0
2·5	—		6·5 (±1·0)c	92·3
5	15·7 (±3·1)b	81·5	3·2 (±1·3)c	96·2

[a] Injections of carvacrol were made 10 cm deep with an injector at 30-cm intervals in rows 30 cm apart.
[b] Treated on 20 May 1994.
[c] Treated on 25 November 1994.
[d] Emergence rate; see Section 2.2.1.
[e] Means followed by the same letter in column are not significantly different ($P = 0{\cdot}05$; Tukey's studentized range test).
[f] Protective value; see Section 2.2.1.
[g] Not determined.

TABLE 4
Susceptibility of *Thecodiplosis japonensis* Larvae to Phosphamidon Alone and in Mixture with Carvacrol by Plant Implantation Application

Chemical	*Rate, ml cm*$^{-1}$ *DBH*	*Mortality* (%) (± *SEM*)[a]	
		Experiment I[b]	*Experiment II*[c]
Carvacrol	0·3	10·8 (± 1·5)c	—[d]
Carvacrol + phosphamidon	0·15 + 0·15	95·0 (± 1·2)a	92·3 (± 2·5)b
Phosphamidon	0·3	81·8 (± 6·1)b	83·4 (± 1·6)c
Untreated	—	1·8 (± 0·1)c	2·0 (± 0·6)a

[a] Means followed by the same letter in column are not significantly different ($P = 0{\cdot}05$; Tukey's studentized range test).
[b] Treated on 15 June and determined on 20 September 1994.
[c] Treated on 20 June and determined on 25 September 1995.
[d] Not determined.

Table 2. Of the terpenoids tested, carvacrol revealed the most potent larvicidal activity.

3.2 Field studies

The efficacy of carvacrol against *T. japonensis* larvae was investigated by soil injection (Table 3). In a field test at Pyungchang-Gun, carvacrol was highly effective against *T. japonensis* larvae at a rate of 5 ml as compared with control. Therefore, titration studies were performed at heavily damaged sites in Kangnung-Si. Significant differences in emergence rates were observed for each treated plot. Over 90% control was achieved in the plots treated with 2·5 and 5 ml of carvacrol, whereas treatment with 1 ml produced 59% control.

In a test with trunk implantation in 1994 (Table 4), phosphamidon (0·3 ml cm^{-1} DBH) gave outstanding control of *T. japonensis* (81·8% mortality), whereas no significant efficacy (10·8% mortality) was produced by carvacrol alone (0·3 ml cm^{-1} DBH). However, a mixture (0·15 + 0·15 ml cm^{-1} DBH) of carvacrol and phosphamidon was highly effective (95·0% mortality), more so than phosphamidon alone (0·3 ml cm^{-1} DBH). Similar results were also obtained from the 1995 experiments. No leaf burn or other symptom of phytotoxicity was observed for any of the chemical treatments.

4 DISCUSSION

Various compounds, including phenolics, terpenoids and alkaloids, exist in plants and jointly or independently contribute to their insecticidal activities.[13] Many *Thujopsis* species are rich in terpenoids.[14–16] In our laboratory study with field-collected *T. japonensis* larvae, carvacrol with potent larvicidal activity was isolated from the crude oil from *Thujopsis* sawdust. This is the first report on insecticidal activity of carvacrol against this insect species, although investigations have shown that this plant-derived material has insecticidal activity against termites[17–19] and stored-insect pests[19] and antimicrobial[20] and rodent-repellent effects.[10]

Ahn *et al.*[10] pointed out that carvacrol might be used as a pest-control agent in limited spaces such as storage bins, greenhouses or buildings, because of its high volatility. In our study with soil and impregnated filter paper applications, carvacrol was highly effective. These results indicate that carvacrol might have both contact and fumigant action. It has been reported that the mode of action of carvacrol against termites and stored-product insects is attributable to both contact and fumigant effects.[19]

Control of the gall midge in Korea is most commonly dependent upon trunk implantation of systemic insecticides such as phosphamidon.[5] Although much success has been achieved using systemic insecticides for the control of insects on coniferous trees when implanted into the trunk,[21] these compounds have attendant problems such as human health hazards. Therefore, much attention has been focused on alternative management methods without, or with reduced, use of conventional insecticides. In the present study with trunk implantation, a mixture of carvacrol and phosphamidon revealed much more potent larvicidal activity than phosphamidon alone, in spite of little or no insecticidal activity of carvacrol alone. These results suggest that carvacrol may promote rapid translocation of phosphamidon into pine trees or prevent rapid degradation of this insecticide in *T. japonensis* larvae and trees. It is possible that a mixture of insecticides may increase insecticidal activity, delay the development of insecticide resistance, or control insect species resistant to single insecticides.

It has been well acknowledged that certain plant-derived extracts and phytochemicals are potential alternatives to insecticides,[6–8,22] based upon the facts that they have selectivity towards the natural enemies of pests, act in many ways on various types of pest complex, and may be applied to the plant in the same way as other agricultural chemicals. In addition, plant-derived materials are found to be highly effective on insecticide-resistant insect pests.[23–26] Carvacrol has low toxicity[27] and caused no mutagenicity, when tested against four strains of *Salmonella typhimurium* Castell. & Chalm.[19] Based upon our data and earlier findings, good potential exists for the control of field populations of *T. japonensis* with a reduced level of phosphamidon when applied with carvacrol to soil in late November and when a mixture of phosphamidon and carvacrol is implanted in mid-June. An average of 4000 tonnes of sawdust per year is produced as a by-product of the timber industry, because *T. dolabrata* is an important timber species in Japan.[28] Thus there is an adequate source of the crude oil available.

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